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Influence of *env* and Long Terminal Repeat Sequences on the Tissue Tropism of Avian Leukosis Viruses

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Adsorption and penetration of retroviruses into eucaryotic cells is mediated by retroviral envelope glycoproteins interacting with host receptors. Recombinant avian leukosis viruses (ALVs) differing only in envelope determinants that interact with host receptors for subgroup A or E ALVs have been found to have unexpectedly distinctive patterns of tissue-specific replication. Recombinants of both subgroups were highly expressed in bursal lymphocytes as well as in cultured chicken embryo fibroblasts. In contrast, the subgroup A but not subgroup E host range allowed high levels of expression in skeletal muscle, while subgroup E but not subgroup A envelope glycoproteins permitted efficient replication in the thymus. A subgroup B virus (RAV-2), like the subgroup E viruses, demonstrated a distinct bursal and thymic tropism, further supporting the theory that genes encoding receptors for subgroup B and E viruses are allelic. The source of long terminal repeats (LTRs) or adjacent sequences also influenced tissue-specific replication, with the LTRs from endogenous virus RAV-0 supporting efficient replication in the bursa and thymus but not in skeletal muscle. These results indicate that ALV *env* and LTR regions are responsible for unexpectedly distinctive tissue tropisms.

Avian leukosis viruses (ALVs) constitute a group of closely related retroviruses that are transmitted in chickens by endogenous proviral DNA as well as by exogenous infection. Naturally occurring ALVs are classified into five subgroups (A, B, C, D, and E) on the basis of viral envelope gp85 determinants which use specific host-encoded receptors. Subgroup A, B, C, and D envelope antigens are found in isolates of exogenous viruses, while subgroup E envelope antigens are characteristic of endogenous viruses (25). Receptors for subgroup B and E (and possibly D) viruses are thought to be encoded by a series of alleles at the *tv-b* locus, with each allele encoding a receptor with unique affinities for B and E viruses (7). Receptors for A virus are encoded at the *tv-a* locus. ALVs also differ in the transcriptional control elements found within their long terminal repeat (LTR) sequences, with the LTR of the endogenous virus RAV-0 promoting less-efficient virus growth in cultured chicken embryo fibroblasts (CEFs) than the LTRs of exogenous viruses (26). To test for effects of envelope and LTR sequences on the tissue tropisms of ALVs, we have evaluated the relative growth of a series of in vitro-constructed ALVs in a variety of tissues.

Recombinant viruses were constructed from the molecularly cloned DNAs of RAV-1 and RAV-0 (Fig. 1). RAV-1 is a subgroup A virus isolated from stocks of the Bryan high-titer strain of Rous sarcoma virus, in which it serves as a helper for the defective sarcoma virus (19). It induces a high incidence of B-cell lymphoma and a low incidence of a variety of other neoplasms in day-old inoculated chickens (18). RAV-0 is a subgroup E virus encoded by the endogenous virus which resides at *ev* 2 (1). It appears to be very low in pathogenicity, since little disease has been observed to follow RAV-0 inoculation of susceptible day-old chicks (6, 15). Viruses were recovered from constructed DNAs by transfection into turkey cells (12) and were verified for subgroup by testing for interference with superinfection by RAV-0 or RAV-1 pseudotypes of Rous sarcoma virus (29). The growth of the test viruses on K28 CEFs was determined; CEFs were infected with undiluted virus stocks and passed three times (approximately 10 days in culture) before being harvested by trypsinization. Fibroblasts were then assayed for the amount of total protein (5) and for the amount of virus capsid protein p27 by using a solid-phase enzyme-linked immunosorbent assay (22). The results of these tests indicated that the viruses varied in their growth potential on CEFs, with RAV-1 growing the best and RAV-0 growing the least well (Fig. 1). In general, the more RAV-1 information a recombinant contained, the better it grew on CEFs.

To evaluate tissue tropisms, groups of day-old K28 chicks were inoculated intravenously with 0.2 ml of undiluted stocks of the various recombinants and parental viruses. These stocks contained from 4 to 66% of the amount of p27 found in a standard stock of RAV-2 (6). K28 chickens can be infected by both subgroup A and E viruses (14, 17). Groups of infected chicks and uninfected control chicks were housed under separate quarantine. At 4, 6, or 8 weeks postinoculation, tissues were harvested and quick-frozen for later analysis. Tissues were harvested at these three times to verify that each virus had undergone maximum spread within the test period. Tissues were selected to represent hematopoietic tissues, reproductive tissues, various visceral organs, lung, skeletal muscle, and brain.

Role of subgroup E and subgroup A *env* sequences in tissue tropisms. Results of p27 assays for the test viruses with exogenous LTRs are presented in the upper half of Table 1, which focuses on four tissues associated with high levels or distinctive patterns of viral replication. Each of the subgroup E recombinants replicated well in the bursa and thymus, with negligible replication occurring in skeletal muscle and very low levels of replication in the gonads. The subgroup A viruses also replicated well in the bursa. However, in contrast to the subgroup E viruses, both of the subgroup A viruses were associated with high p27 expression in skeletal muscle and gonads and with variable expression in the thymus. WF181, a subgroup A virus with *gag* and *pol* of RAV-0, replicated poorly in the thymus, whereas RAV-1 replicated well in the thymus.

Tissue distribution of subgroup A and subgroup B viruses in

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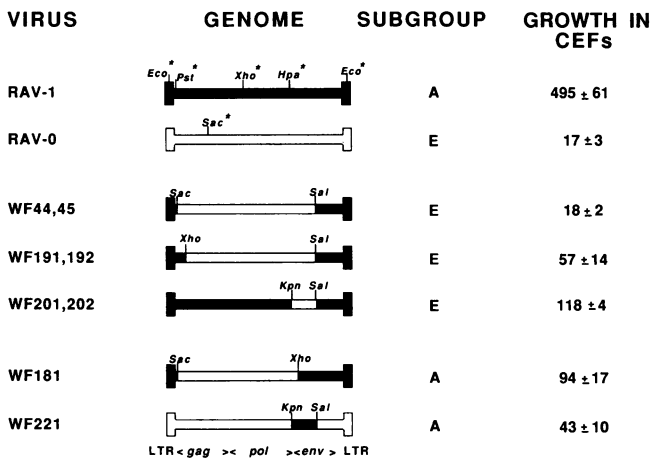

FIG. 1. Genomes of parental and recombinant viruses and their relative growth in CEFs. Symbols: ▭, portions of the genome derived from pRAV-0, a closed circular RAV-0 DNA cloned at the *Sal*I site in *env* into the *Sal*I site of pBR322; ▬, sequences derived from pRAV-1, a closed circular RAV-1 DNA cloned at the *Sac*I site near the 5′ LTR into a *Sac*I site of a derivative of pBR322; *, nonconserved sites used to verify the origins of fragments are indicated in the parental genomes. The genomes of the recombinant viruses indicate the conserved restriction endonuclease sites used in the construction. A more complete listing of the restriction endonuclease sites in pRAV-1 and pRAV-0 can be found in Robinson et al. (16). WF44,45, WF191,192, and WF201,202 designate duplicate viruses recovered from independently derived constructs. As the members of these pairs had indistinguishable phenotypes, pairs are referred to as the WF40s, the WF190s, etc. *Eco*, *Eco*RI; *Hpa*, *Hpa*I; *Kpn*, *Kpn*I; *Pst*, *Pst*I; *Sac*, *Sac*I; *Sal*, *Sal*I; *Xho*, *Xho*I. Growth in CEFs is represented as the titer of viral capsid protein p27 in lysates of virus-infected CEFs at 50 μg/ml relative to the concentration of p27 in a RAV-2 standard stock × 10^4. Means ± standard errors were obtained from two independent infections of K28 CEFs. The enzyme-linked immunosorbent assay for detection of p27 is described in Table 1, footnote *b*.

line 6_3. Since formation of subgroup E recombinants between RAV-1 or WF181 and *ev* 1, an endogenous viral locus present in K28, could have broadened the tissue tropism of RAV-1 or WF181, the tropism of RAV-2, a subgroup B virus, was compared with that of RAV-1 in line 6_3 chickens. Line 6_3 chickens are susceptible to infection by B but not E virus, and thus formation of subgroup E recombinants in this line should not influence tissue tropism. In Table 2, patterns of RAV-2 and RAV-1 replication in tissues from line 6_3 chickens are compared. The relative levels of RAV-2 and RAV-1 replication in the different tissues reflected patterns which had been observed for the subgroup A and E recombinants described above. For example, RAV-2 consistently replicated better in the thymus and bursa than in muscle, whereas RAV-1 replicated better in the bursa and muscle than in the thymus. Similar patterns of RAV-1 and RAV-2 replication were also observed in two other subgroup A and B virus-susceptible but subgroup E virus-resistant chicken pedigrees, line 0 and SPAFAS flock 39 (data not shown). A more efficient RAV-1 infection of the bursa relative to the thymus has also been noted in Sc chickens (3).

Role of RAV-0 LTR and immediately adjacent sequences in tissue tropisms. In the lower half of Table 1, the tissue distribution associated with RAV-0 and WF221, a virus which is identical to RAV-0 except for sequences encoding the subgroup A host range, are compared. RAV-0 replicated well in the bursa and thymus, while WF221 replicated well only in the bursa. The replication of RAV-0 in the bursa and thymus is in keeping with the tissue tropism of the other subgroup E viruses and indicates that the endogenous RAV-0 LTR is able to function relatively well in these tissues. In contrast, the poor replication of subgroup A WF221 in muscle, a tissue which supports vigorous replication of other subgroup A viruses, suggests that the RAV-0 LTR (and possibly other adjacent sequences; Fig. 1) is not effective in this tissue. When K28 chicks infected with WF221 were sacrificed earlier in the course of infection, at no time was efficient replication observed in skeletal muscle or gonadal tissue. Thus the limited distribution of this subgroup A virus is not the result of viral clearance from infected tissues but most likely reflects limitations on viral replication or spread imposed by the RAV-0 LTR or adjacent sequences. Bursal cells have been noted to express a tissue-specific factor(s) which influences the transcriptional activity of exogenous LTRs (13). Whether this same factor(s) also controls the activity of the RAV-0 LTR in the bursa or thymus is not known.

Other tissues. Three recombinants (the WF40s, WF181, and WF221) were tested in an initial extensive screening of tissues. Each of the recombinants replicated about 10-fold less efficiently in the spleen than in the bursa. In the lungs, kidneys, and pancreas, WF181 replicated to levels comparable to that observed in the gonads. The WF40s and WF221 replicated less well than WF181 in each of these tissues. Replication in the brain, liver, and erythrocytes was minimal.

Implications for tissue-specific expression of receptors for ALVs. Distinct patterns of tissue-specific replication associated with viruses differing only in subgroup specificity imply that differences in replication are due to a receptor-mediated step. Earlier studies on the comparative tropisms of subgroup A and B ALVs have suggested that subgroup B but not subgroup A viruses efficiently infect bone marrow cells and osteocytes, that subgroup B viruses replicate better than subgroup A viruses in yolk sac macrophages (10), and that subgroup A but not B viruses productively infect such endocrine epithelial cells as are found in the adrenal cortex and thyroid (8). Expression of a subgroup A ALV in the skeletal muscle of inoculated avian embryos has also recently been described (9). Our results, taken together with these, suggest that receptors for subgroup A virus have a broad distribution in connective, endocrine, and reproductive tissues and a more limited distribution in hematopoietic tissues. In contrast, the polymorphic family of receptors for viruses of subgroups B and E (7) appear to be broadly distributed in hematopoietic tissues.

Neither our study nor previous studies on tissue-specific virus expression can be used to exclude the presence of virus receptors on tissues that scored negative for all tested viruses. In such tissues, virus could have undergone a nonproductive infection following adsorption and penetration. Indeed, this appears to be so in erythrocytes, which contain exogenous proviral DNA (data not shown; 3) but which express little or no virus. Also, differences in tissue-specific expression of virus do not necessarily reflect absolutes in terms of expression of a receptor, as differences in receptor density or tissue-specific modification of a receptor could influence susceptibility to a virus infection.

Implications for virus-host interactions. The unexpectedly distinctive tissue tropisms we have observed for ALVs with different *env* and LTR sequences need to be considered in future interpretations of ALV-host interactions which influence disease induction and immune response.

TABLE 1. LTR and *env* influences on the tissue tropism of ALV recombinants

LTR source	Subgroup	Virus	No. of wingband of chick	No. of weeks PI[a]	Relative p27 titer[b] by tissue			
					Bursa	Thymus	Muscle	Gonad
RAV-1	E	WF44 and WF45	2997	8	91	33	<1	<1
			3031	8	345	244	<1	2
			3047	8	95	312	<1	<1
			3048	8	125	<1	<1	1
			3069	8	476	87	<1	1
		3105	8	122	132	<1	1	
		WF191 and WF192	9112	4	145	81	<1	2
			9117	6	62	77	<1	2
			9114	8	120	54	<1	<1
			9121	8	14	2	<1	<1
			9122	8	73	48	<1	1
		WF201 and WF202	9098	6	147	769	<1	3
			9102	6	65	111	<1	2
			9100	8	281	291	<1	2
			9104	8	137	182	2	5
			9108	8	66	80	<1	3
	A	WF181	3231	8	250	2	91	18
			3233	8	185	2	42	21
			3251	8	667	20	172	139
			3253	8	377	6	122	40
			3267	8	208	1	74	18
			3269	8	156	5	111	65
		RAV-1	9170	4	135	111	125	147
		9174	4	104	385	223	93	
			9172	6	446	161	172	133
			9180	6	83	34	119	54
			9176	8	100	54	231	105
RAV-0	E	RAV-0	9079	6	31	51	1	<1
			9090	6	16	50	<1	<1
			9072	8	18	32	1	<1
			9076	8	10	18	<1	<1
			9087	8	59	27	<1	2
	A	WF221	3303	8	147	5	1	3
			3319	8	286	7	5	3
			3367	8	172	<1	1	2
			3383	8	139	7	<1	2
			3415	8	87	3	<1	2
Uninfected control		9153	4	<1	<1	<1	<1	
			9158	4	<1	1	<1	<1
			9155	8	<1	<1	<1	<1
			9157	8	<1	<1	<1	<1

[a] PI, Postinoculation. K28 chicks were inoculated intravenously with 0.2 ml of undiluted virus stock at 1 day of age. Tissues were collected at the indicated times.

[b] Values represent the concentration of virus capsid protein p27 in samples diluted to 50 μg of tissue protein per ml relative to the concentration of p27 in a RAV-2 culture medium × 10^4. Tissue homogenates were prepared by grinding in the presence of 0.8% deoxycholate, 0.8% Nonidet P-40, 10 μg of aprotinin per ml, and 2 mM phenylmethylsulfonyl fluoride. The amounts of p27 were determined by using an enzyme-linked immunosorbent assay. The initial coat consisted of a monoclonal antibody for capsid p27 antigen. Microtiter wells were blocked with 2% bovine serum albumin in carbonate buffer and incubated with samples diluted in phosphate-buffered saline containing 1% bovine serum albumin and 0.1% Tween 20 (Sigma Chemical Co., St. Louis, Mo.). Bound p27 was then detected by using a rabbit anti-capsid antiserum followed by an alkaline phosphatase conjugate of goat anti-rabbit immunoglobulin G (Cooper Biomedical, Inc., Cochranville, Pa.) and finally by the *para*-nitrophenyl phosphate substrate for alkaline phosphatase. A_{405} was determined by using a Bio-Tek EL-307.

Thymus. The thymotropism associated with subgroup B and E viruses could be a major contributing factor to the immunosuppression associated with some subgroup B ALVs (20, 23). We have found that the thymotropic virus RAV-2 does not cause generalized immunosuppression but does very effectively induce tolerance (work in progress).

Skeletal muscle. The subgroup A virus muscle tropism could in part explain the occurrence of a muscle disease induced by recombinant ring-necked pheasant viruses with subgroup A but not subgroup F envelope antigens (21). The finding of high levels of replication in the skeletal muscle of chicks inoculated after hatching (Tables 1 and 2) is intriguing because postembryonic skeletal muscle growth involves cell enlargement rather than cell division (11, 24) and host cell DNA replication is thought to be required for integration and expression of viral genes (27, 28).

Bursa. High levels of viral replication were detected in the bursas of line 6_3 chickens (Table 2), a line known for its resistance to ALV-induced lymphoma induction (4). Also, in the lymphoma-susceptible line K28, the levels of viral replication in the bursa at 1 to 2 months of age did not correlate well with lymphoma induction (6, 15). However, the extent

TABLE 2. Replication of RAV-1 and RAV-2 in tissues of line 6_3 chickens[a]

Virus (subgroup)	No. of wingband of chick	Relative p27 titer[b] by tissue or serum				
		Bursa	Thymus	Muscle	Gonad	Serum
RAV-2 (B)	143	338	338	34	68	625
	147	222	286	61	74	625
	153	312	403	14	37	1,053
RAV-1 (A)	135	333	100	278	38	NT
	149	352	30	142	45	77
	163	312	95	142	91	244
None	165	12	15	5	10	18

[a] Chicks were inoculated intravenously with 0.2 ml of undiluted virus stock at 1 day of age. Tissues were collected 3.5 weeks later.

[b] Amount of p27 relative to that in a RAV-2 standard stock $\times 10^4$. Tissue homogenates were tested as described in Table 1, footnote *b*. The presence of p27 in tissues from the uninfected control is attributed to *gag* expression by the endogenous virus locus *ev* 3, present in line 6_3 (2). NT, Not tested.

of viral replication in the bursas of K28 chicks during the first 3 weeks post-hatch does correlate with lymphoma induction (6).

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